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Immunostimulatory complexes containing *Eimeria tenella* antigens and low toxicity plant saponins induce antibody response and provide protection from challenge in broiler chickens

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ABSTRACT

Immunostimulating complexes (ISCOMs) are unique multimolecular structures formed by encapsulating antigens, lipids and triterpene saponins and are one of the most successful antigen delivery systems for microbial antigens. In the current study, both the route of administration and the antigen concentration of ISCOMs, containing *Eimeria tenella* antigens and saponins from native plants, were evaluated in their ability to stimulate humoral immunity and to protect chickens against a challenge infection with *E. tenella*. Broiler chickens were immunized with ISCOM preparations containing *E. tenella* antigens and the purified saponins Gg6, Ah6 and Gp7 isolated from *Glycyrrhiza glabra*, *Aesculus hippocastanum* and *Gipsophila paniculata*, respectively. The effects of the route of administration, dose of antigen and type of saponin used for construction of ISCOMs were evaluated for ability to stimulate serum IgG and IgM and to protect chickens against a homologous challenge. A single intranasal immunization was the most effective route for administering ISCOMs although the in ovo route was also quite effective. Dose titration experiments demonstrated efficacy after single immunization with various ISCOM doses but maximum effects were observed when ISCOMs contain 5–10 μg antigen. Immunization of birds by any of the three routes with *E. tenella* antigens alone or antigens mixed with alum hydroxide adjuvant resulted in lower serum antibody and reduced protection to challenge relative to immunization with ISCOMs. Overall the results of this study confirm that significant immunostimulation and protection to challenge are achieved by immunization of chickens with ISCOMs containing purified saponins and native *E. tenella* antigens and suggest that ISCOMs may be successfully used to develop a safe and effective vaccine for prevention of avian coccidiosis.

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1. Introduction

Avian coccidiosis, caused by obligate intracellular parasites belonging to various species of the genus *Eimeria*, is estimated to cost the world-wide poultry industry several billion dollars annually in mortality and bird productivity (Dalloul and Lillehoj, 2005). The disease has been primarily controlled by use of chemicals, but their extensive use over the past 40 years has resulted in the development of drug resistance by these parasites (McDougald, 1993; Li et al., 2004, 2005). Due to food safety concerns and the cost of new drug development,

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recent emphasis has centered on elicitation of protective immune response to parasite infection by development and effective use of live or inactivated parasite vaccines (Danforth et al., 1997; Danforth, 1998; Dalloul and Lillehoj, 2005). Vaccines constructed with purified coccidian antigens or recombinant antigens prepared from cloned Eimeria DNA sequences may induce comparatively high levels of specific immune response and provide protective immunity against coccidial infection (Danforth et al., 1989; Jenkins, 1998). However, an improvement on the degree of protective immune response elicited by these antigens is needed for practical use of such vaccine preparations in the poultry industry.

Nearly all subunit vaccine proteins, whether they may be recombinant or purified natural products, are comparatively weak immunogens and require repeated boosting and use of adjuvants to induce protective immune responses. These applications are not acceptable to the poultry industry because of the cost involved for repeated administrations or lack of government clearance for oil-based adjuvants used in food animals. The development of other novel types of antigen delivery that will elicit greater immune response is needed.

Immunostimulating complexes (ISCOMs), are one of the most successful antigen delivery systems for microbial, viral and parasite antigens Morein and Simons, 1985; Kensil, 1996; Agraval et al., 2003; Morein and Abasugra, 2004). In a recent study, ISCOMs formulated with isolated *E. tenella* antigens and low toxicity saponins, isolated from a number of plants indigenous to Kazakhstan, were more effective in stimulating immunity and protecting against challenge infection than *E. tenella* antigens alone (Berezin et al., 2008). Similarly, immunization of broiler breeder chickens with ISCOMs containing *E. tenella* sporozoite antigens and the commercial saponin, Quil A (Kensil et al., 1991), was effective in stimulating immune responses and protecting against challenge infection (Garcia et al., 2008).

In the current study, the route of administration, the type of saponin and the antigen concentration of ISCOMs was evaluated in their ability to stimulate humoral immunity and to protect chickens against a challenge infection.

2. Materials and methods

2.1. Host and parasites

Chickens (1-day-old Titan broilers) were purchased from local hatcheries. Feed and fresh water were given ad libitum. Feed was purchased from a local market. It was comparatively crude and was not enriched with proteins, vitamins, probiotics or other additives and nor did it contain anticoccidials. Before any experimental procedures were initiated, chickens were allowed to acclimate for 3–7 days. Chickens with a negative antibody titer for *E. tenella* antigens were transferred to separate housing for experimental procedures. Chickens used in all experiments were handled according to the guidelines of the Institutional Animal Care and Use Committee.

E. tenella (strain AK-1) a single oocyst-derived line isolated from naturally infected chickens in Almaty, Kazakhstan, was maintained by serial passage in 10- to 14-day-old chicks (Berezin et al., 2008). Oocysts were collected from the cecae of infected birds, cleaned and sporulated as described earlier (Tomley, 1997; Fetterer and Barfield, 2003).

2.2. Isolation of antigens

E. tenella oocysts, sporozoites and merozoites were isolated and purified as previously described (Tomley, 1997; Fetterer and Barfield, 2003). *E. tenella* antigens were prepared by detergent extraction mixture of oocysts, sporozoites and merozoites as previously described (Berezin et al., 2008). Viral antigens were prepared from purified concentrated avian influenza virus, strain A/FPV/Rostok/34 (H7N1) grown in 9-day-old chicken embryos. External viral antigens HA + NA were isolated by non-ionic detergent extraction and purified from internal "core" proteins by centrifugation and from detergent by dialysis.

2.3. Preparation of saponins

Saponins for ISCOMs formation were isolated from three native plants collected in mountainous regions of Kazakhstan: *G. glabra*, *A. hippocastanum* and *G. paniculata*. Crude saponins were extracted from roots and seeds by 95% ethanol extraction and partially purified from low weight substances by extensive dialysis against phosphate-buffered saline. Plant extracts were lyophilized and fractionated by HPLC as previously described (Berezin et al., 2008). HPLC fractions containing highest concentration of saponins were further purified by repeated HPLC fractionation in a linear gradient from 0.1% TFA in water to 80% ACN in water with 0.1% TFA. Partially purified saponins Gg6 (isolated from G. *glabra*), Ah6 (isolated from *A. hippocastanum*), Gp7 (isolated from *G. paniculata*) and a commercial preparation of saponin Quil A (Isconova, Sweden) were used for ISCOMs formation. Before use in immunological experiments, toxicity of saponin preparations was characterized as previously described (Berezin et al., 2008).

2.4. ISCOMs

ISCOMs were prepared by a dialysis technique (Berezin et al., 2008). To verify their characteristic structure, samples of ISCOMs were examined by electron microscopy using negative stain with instrumental magnification of 60,000 (Ozel et al., 1989), analyzed by electrophoresis in 12.5% SDS-polyacrylamide gel (Laemmli, 1970) and amount of protein was determined (Bradford, 1976).

2.5. Effect of route of antigen administration and antigen dose on antibody titer

To assess the effect of route of administration on antibody titer, chickens (7-day old, 12 per group) were immunized by the intranasal or oral route with ISCOMs containing *E. tenella* antigens and various saponins (Gg6, Ah6, Gp7, and Quil A), *E. tenella* antigens alone or antigens mixed with alum hydroxide adjuvant at a dose

of 10 μg/chicken. Titers of IgG and IgM antibodies were determined in sera of 21-day-old chickens. For in ovo immunizations, 18-day-old chicken embryos (12 per group) were immunized into the chorion-allantoic cavity by inoculation with vaccine preparations described above at a dose of 10 μg antigen in 0.2 ml saline. A control group was immunized with 0.2 ml saline (placebo). Embryos were placed into incubator and chicks hatched in a normal manner. To assess the effect of antigen dose on antibody titer, chicks (12 per group) were immunized with vaccine preparations as described above with doses of 0.2, 1.0, 5.0 and 10 μg antigen per chick delivered intranasally.

At 14 days post-immunization (intranasal and oral routes) or 14-day-old chicks (in ovo immunization), blood was collected by heart puncture followed by cervical dislocation. Titers of IgG and IgM serum antibodies were obtained from serum pooled from 12 chicks per group and determined using a conventional ELISA assay. Briefly, flat-bottom, 96-well microtiter plates were coated with 200 μl per well of 10 μg/ml *E. tenella* antigen (same antigen as used for vaccination) dissolved in 0.05 M Tris–HCl, pH 7.4. Wells were blocked with Tris–HCl containing 10% horse serum with 0.05% Tween-20 and incubated with serum samples for 2 h at 37 °C. The plate was washed with Tris–HCl buffer containing Tween-20 and incubated with anti-chicken immunoglobulin G peroxidase conjugate (Sigma, St. Louis, MO). Peroxidase-labeled antibodies were detected using Sigma Fast™ o-orthophenylendiamine dihydrochloride tablet set (Sigma) as substrate. Optical density (OD) was measured at 492 nm on a multichannel spectrophotometer. A sample was considered to be positive when the OD of sample was more than twice that of the negative control. Antibody titers were determined as the maximum positive dilution of serum samples.

2.6. Challenge experiments

Chickens were infected with 50,000 oocysts per bird by oral gavage 14 days post-immunization using immunization groups as described above for determining antibody titer. Chickens were weighed daily. Six days after infection, blood was collected by heart puncture and chickens were euthanized by cervical dislocation. The serum concentrations of carotenoids and nitrate–nitrite were determined as previously described (Allen, 1987, 1997) and were expressed as μg/ml of triplicate measurements of serum pooled from six birds per treatment group. The oocyst counts were determined on triplicate counts on samples pooled from six birds in each treatment group. Chickens were weighed daily on an electronic balance to 0.1 g. Average daily weight gains were determined as previously described (Berezin et al., 2008) and represent the mean of 12 chicks per treatment group. The relationship between

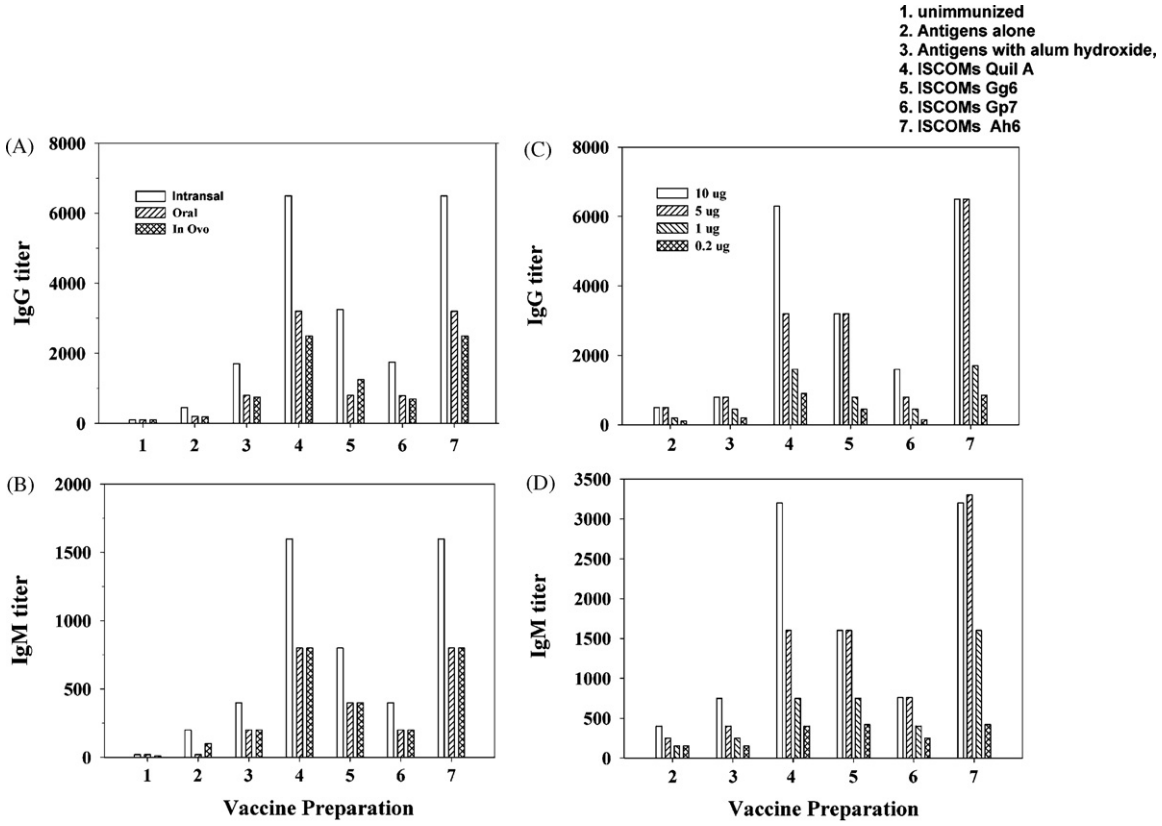

Fig. 1. Titers of IgG (A) and IgM (B) in chicken sera after intranasal, oral and in ovo immunization with ISCOMs containing 10 μg *E. tenella* antigen and various saponins. Titers of IgG (C) and IgM (D) antibody in chicken sera after intranasal immunization with 0.2, 1.0, 5.0 or 10 μg antigen per chick. Values on *y*-axis are titers of sera pooled from 12 chickens per treatment group. Values on *x*-axis represent the immunization group.

log of the antigen dose and response in weight gain, oocysts output, and plasma carotenoid and nitrate–nitrite levels after *E. tenella* challenge was analyzed by linear regression (Sigma Plot, Systat Software Inc., San Jose, CA).

2.7. Immunization with irrelevant antigens

Seven-day-old chickens (12 per group) were immunized intranasally with ISCOMs containing isolated influenza virus antigens (HA + NA) and various saponins (Gg6, Ah6, Gp7, and Quil A) at a dose of 10 μg/chicken and isolated viral antigens alone at a dose of 10 μg/chicken. Chickens (12 per group) were also immunized with ISCOMs containing isolated native *E. tenella* antigens and saponins Gg6, Ah6, Gp7, Quil A at a dose of 10 μg/chicken and *E. tenella* antigens alone at a dose of 10 μg/chicken. At 14 days post-immunization chickens were infected with 50,000 oocysts per bird by oral gavage. Six days after infection, blood was collected by heart puncture and chickens were euthanized by cervical dislocation. The serum concentration of carotenoids and nitrate–nitrite were measured in serum pooled from six birds per treatment group as described above. The oocyst number was determined on triplicate counts of samples pooled from six birds in each treatment group. Daily weight gain was not measured in this experiment.

3. Results

3.1. Antibody titer

All three routes of administration of ISCOMs containing *E. tenella* antigens stimulated comparatively high serum levels of IgG (Fig. 1A) and IgM (Fig. 1B). The highest titers of both IgG and IgM were observed following immunization with ISCOMs containing saponins Gg6, Ah6 and Quil A. Antibody titers, from chicken immunized with ISCOMs exceeded the titer of chickens immunized with isolated *E. tenella* antigens alone or mixed with alum hydroxide. Intranasal immunization resulted in higher IgG and IgM antibody titers in comparison with oral or in ovo vaccination. In ovo immunization produced antibody titers similar to those observed with the oral immunization.

The effect of intranasal immunization with ISCOMs, containing a range of antigen concentrations, on serum antibody levels was evaluated (Fig. 1C and D). Generally, the serum antibody levels observed after immunization were related to amount of antigen incorporated into each

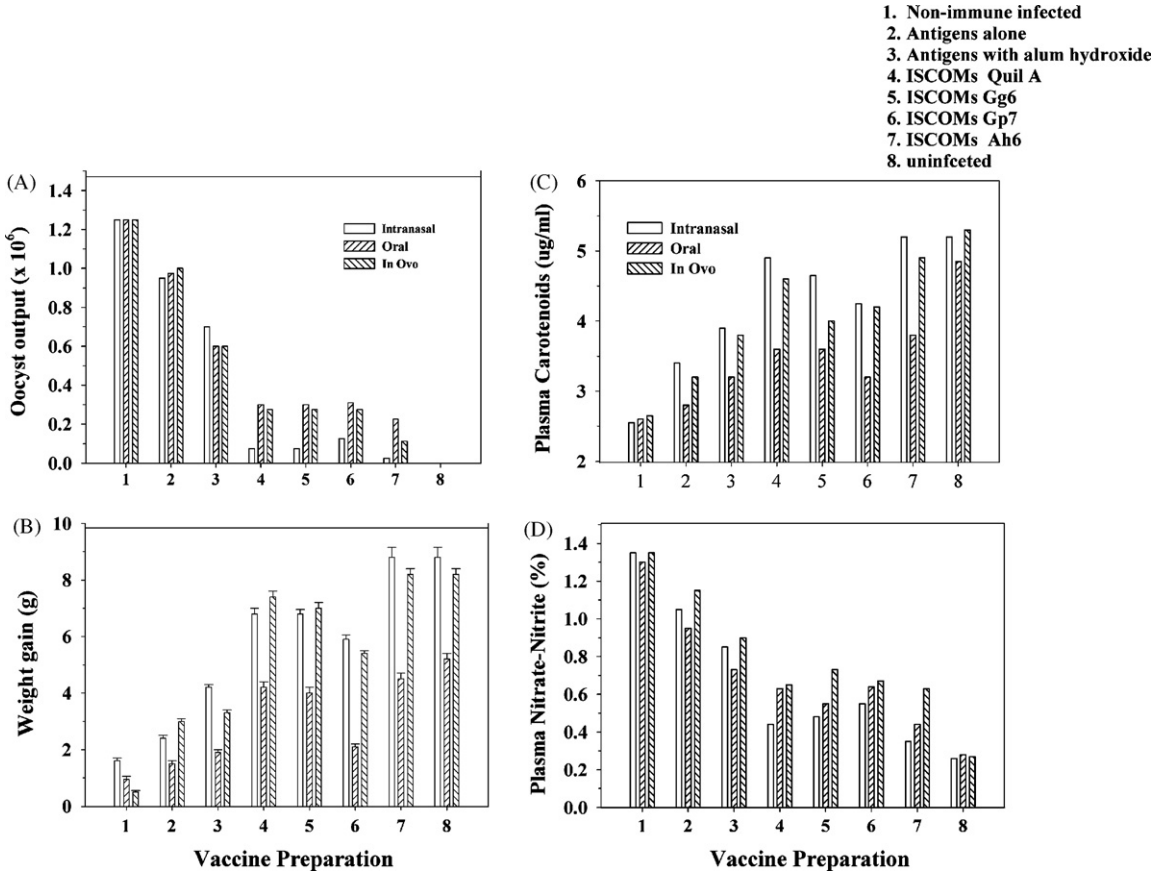

Fig. 2. The changes in oocyst output (A), weight gain (B), plasma carotenoids (C) and nitrate–nitrite (D) levels after intranasal, oral and in ovo immunization followed by challenge with *E. tenella* oocysts. Values on the *y*-axis represent triplicate measurements (A, C and D) on pools of six chicks per group. For weight gain (B) the values are means of 12 chicks per group and error bar represents the SEM. For all graphs the numbers on the *x*-axis indicate immunization groups.

ISCOM preparation. The highest antibody titers were observed with 10 μg of antigen with either Quil A or Ah6 as the saponin. ISCOM preparations stimulated production of both IgG and IgM antibody in significantly higher titers in comparison with immunization of *E. tenella* antigens alone or antigens mixed with alum hydroxide adjuvant.

3.2. Challenge infections

Immunization of chickens with ISCOMs containing 10 μg of *E. tenella* antigens and various saponins conveyed protection against challenge with *E. tenella* oocysts (Fig. 2). The cecal oocyst content of *E. tenella* infected birds was greatly reduced by intranasal immunization with ISCOM preparations compared to other routes of administration (Fig. 2A). Intranasal immunization with ISCOMs containing *E. tenella* antigen and Quil A, Gg6, or Ah6 resulted in a greater than 90% reduction in the cecal oocyst counts. Immunization with either *E. tenella* antigen alone or antigen mixed with alum reduced the cecal oocysts count by 28 and 44%, respectively. The decrease in average daily weight gain observed in infected unimmunized birds was ameliorated by intranasal, oral or in ovo immunization with ISCOMs containing Ah6 (Fig. 2B). Plasma carotenoid levels in unimmunized birds challenged with *E. tenella* were greatly depressed (Fig. 2C). Intranasal immunization with ISCOMs containing *E. tenella* antigen and either Quil A or Ah6 resulted in carotenoid levels similar to those of uninfected controls. Immunization with either *E. tenella* antigen alone or mixed with alum was less effective than immunization with ISCOMs (Fig. 2C). The plasma levels of nitrate–nitrite were elevated in *E. tenella* infected unimmunized birds (Fig. 2D). With all three routes of administration, ISCOMs were more effective in reducing the levels of plasma nitrate–nitrite compared to immunization with either *E. tenella* antigen alone or antigen mixed with alum. Intranasal immunization with ISCOMs containing *E. tenella* antigen and Ah6 appeared to be the most effective in reducing plasma nitrate–nitrite levels.

After a challenge infection, the daily weight gains, cecal oocyst counts, and plasma carotenoid and nitrate–nitrite levels of ISCOM immunized birds were directly related to the dose of antigen (Fig. 3.) After challenge, cecal oocyst counts (Fig. 3A) were decreased and daily weight gains (Fig. 3B) were increased linearly with the log of the

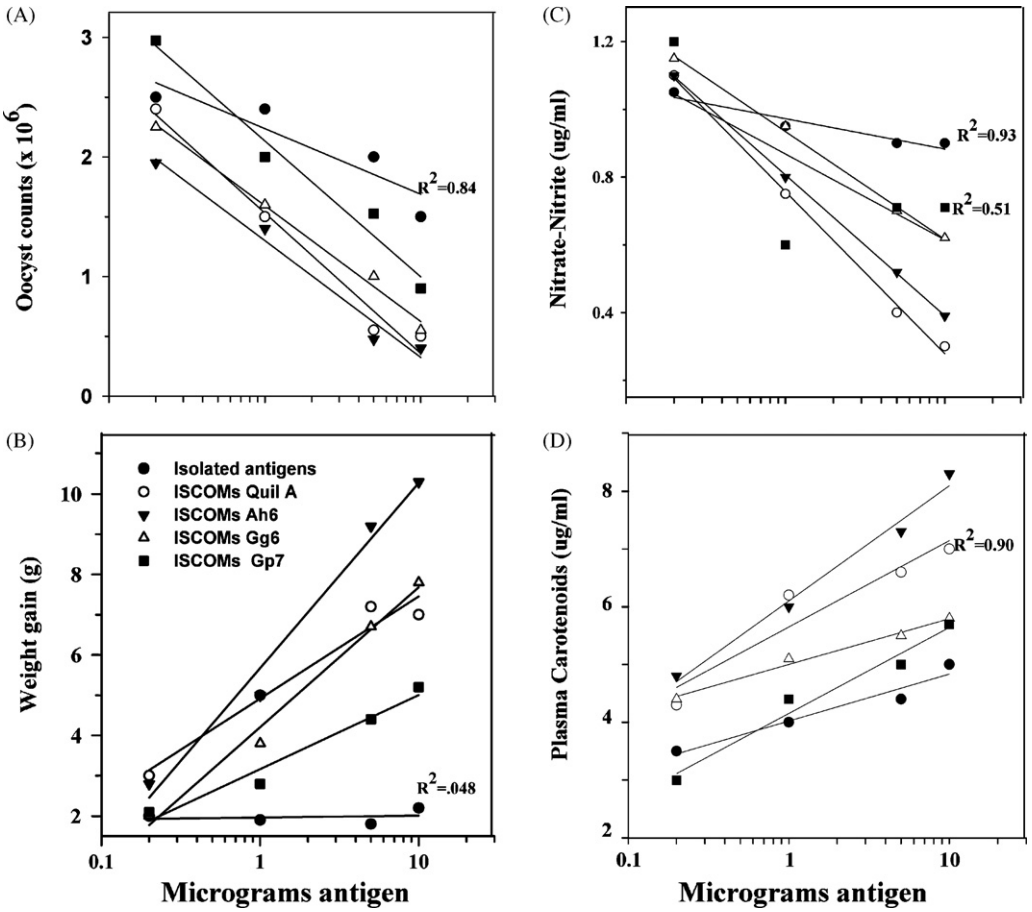

Fig. 3. The relationship, 6 days after challenge with *E. tenella* oocysts, between the logarithm of antigen dose and response of oocyst count (A), weight gain (B), and plasma carotenoid (C) and nitrate–nitrite (D) levels in birds following intranasal immunization with various antigen preparations. Lines were fitted to the data by first-order regression analysis. Unless indicated, the coefficient of determination (R^2) values are greater than 0.96. Values on *y*-axis represent triplicate measurement of samples pooled for six chickens except for weight gain (B) which are means of 12 chickens. Error bars represent SEM.

antigen dose in birds immunized with ISCOM preparations. When birds were immunized with isolated antigen there was no linear relationship ($R^2 = 0.048$) between antigen dose and daily weight gain (Fig. 2B) when birds were immunized with antigen alone (Fig. 3B.). However, there was a linear relationship ($R^2 = 0.89$) between antigen dose and oocyst output although the slope of the line appeared reduced compared to immunization with ISCOMs (Fig. 3A). The decrease in plasma cartenoid levels and the increase in plasma nitrate–nitrite observed in *E. tenella* infected birds was also ameliorated in a dose related manner by intranasal immunization with ISCOM preparations (Fig. 3C and D). Immunization of birds with ISCOMs containing Quil A, Ah6 or Gp6 decreases nitrate–nitrite levels in a dose related manner while immunization with Ah7 resulted in a poor ($R^2 = 0.51$) linear relationship between antigen dose and nitrate–nitrite levels (Fig. 3C). In birds immunized with antigen alone, there was a linear relationship between antigen dose and nitrate–nitrite level ($R^2 = 0.91$) but the slope of the line appeared lower than those obtained with immunizations with ISCOMs. Immunization of birds with all preparations resulted in a significant dose response ($R^2 > 0.92$) of plasma carotenoids (Fig. 3D).

3.3. Immunization with irrelevant antigen

Intranasal immunization with ISCOMs containing 10 μg of viral antigen was ineffective in either reducing oocyst counts or nitrate–nitrite levels or increasing carotenoid levels following challenge with *E. tenella* (Fig. 4).

4. Discussion

Saponins isolated from plants indigenous to Kazakhstan were recently demonstrated to have low toxicity and be capable of forming ISCOMs that provide an attractive antigen delivery system to protect broiler chicks against avian coccidiosis (Berezin et al., 2008). The current study further evaluates the efficacy of ISCOMs as vaccine candidates by assessing the role of route of administration, saponin type and antigen dose in immunization of broiler chickens against a homologous challenge with *E. tenella.* In general, the results indicate that intranasal immunization was the most effective route of vaccine administration. This conclusion is supported by the higher levels of serum antibodies observed following intranasal immunization with ISCOMs compared to in ovo and oral routes. Concurrently, intranasal immunization was most

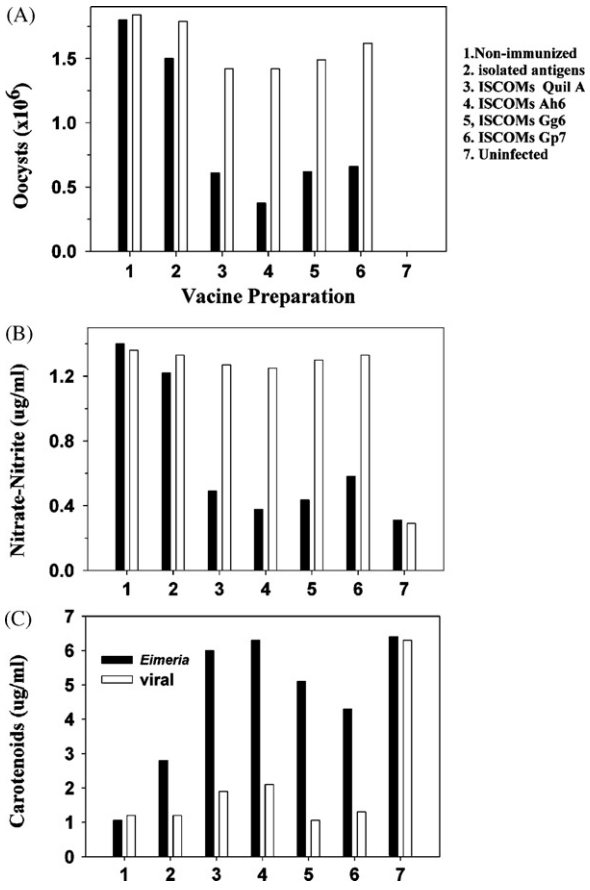

Fig. 4. The effects of intranasal immunization of chickens with an *E. tenella* or viral antigen on oocyst output (A) and plasma nitrate–nitrite (B) and carotenoids levels (C) following an *E. tenella* challenge infection. Values are triplicate measurements of samples pooled from six chickens per treatment group. For all graphs, the numbers on the *x*-axis indicate immunization groups.

effective in conveying protection against homologous challenge as measured by a decrease in cecal oocyst counts, an increase in plasma carotenoids, and decrease in plasma nitrate–nitrite. Plasma carotenoids are decreased during a coccidial infection due to malabsorption caused by parasite invasion of intestinal cells (Allen, 1987), and levels of nitrate–nitrite are increased by infection in relation to inflammatory responses (Allen, 1997) and both serve as sensitive markers of pathology due to infection (Allen and Fetterer, 2001). A mucosal route of immunization may be most effective because many pathogens initiate the infectious process at the mucosal surfaces, and that in these infections, mucosal application of a vaccine is often required to induce a protective immune response. Immunity at the mucosal surfaces is mediated by the mucosa-associated lymphoid tissues (MALT), which represent the largest immune compartment within the body (Holmgren and Cerrkinsky, 2005). Although the intranasal route of administration is a practical way to deliver poultry vaccine, in ovo immunization is currently being used to deliver a live vaccine (Inovocox, Pfizer animal Health) on a large scale and may be acceptable for use with an ISCOM vaccine.

Intranasal immunization with ISCOMS containing *E. tenella* antigen resulted in a dose related response in all infection parameters measured. However, immunization with ISCOMs containing Ah6 appeared to have the steepest dose response curve while responses to ISCOMs containing Quil A were often similar. There was no significant relationship between dose of antigen administered alone and daily weight gain. This observation suggests that immunization with antigen incorporated into ISCOMs is essential for immunity at least as evaluated by weight gain. There was a significant dose–response relationship when chicks were immunized with antigen alone and oocyst output, carotenoid and nitrate–nitrite levels were measured after challenge. However, the slopes of lines calculated for these responses appeared lower than those obtained after immunization with ISCOMs suggesting that antigens when incorporated into ISCOMs are more effective immunogens.

Consistent with our current finding, a recent report indicates that immunization with ISCOMs prepared with Quil A and crude sporozoite antigen protects broiler breeders from an *E. tenella* challenge (Garcia et al., 2008). However, Quil A has greater toxicity than saponins from the native plants used in the present study (Berezin et al., 2008) suggesting that saponins like Ah6 or Gg6 may be better candidates to construct ISCOMs to be used for vaccine delivery. As described above immunization with *E. tenella* antigens alone were much less effective in elevating serum antibody levels or in protecting against challenge infection indicating that incorporating the antigens into ISCOMs enhances the response to the antigen. However, saponins can have non-specific adjuvant properties so that ISCOMs might stimulate a non-specific immune response unrelated to the antigen contained in the ISCOMs. In the current studies, we observed that immunization with ISCOMs containing an irrelevant viral antigen was ineffective in reducing response to challenge infection compared to immunization with ISCOMs containing *E. tenella* antigen suggesting that ISCOMs are essential for development of protection to *E. tenella* challenge. Supporting this observation, Garcia et al. (2008) observed a reduced immune response in broiler breeder chicks immunized with ISCOMs alone compared to ISCOMs containing *E. tenella* antigen.

The current results along with recent findings (Berezin et al., 2008; Garcia et al., 2008) demonstrate the possibility of using ISCOM technology to effectively immunize chickens against infection with avian coccidiosis. However, these studies used crude antigen preparations and only examined protection against homologous challenge with *E. tenella*. To be an effective, practical vaccine treatment for poultry coccidiosis, ISCOM vaccines may need to contain recombinant antigens or combinations of antigens that can provide protection against the several species of *Eimeria* that are important pathogens. Immunization experiments to assess the effect of *Eimeria* antigens to protect against several coccidian species are currently in progress.

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